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# Structure of holo-chaperonin studied with electron microscopy

## Oligomeric cpn10 on top of two layers of cpn60 rings with two stripes each

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A structural model of holo-chaperonin, known as a protein-folding control protein comprising 60 kDa (cpn60) and 10 kDa polypeptides (cpn10), is proposed based on the electron microscopic images of holo-chaperonin from *Thermus thermophilus* and cpn60 from *Paracoccus denitrificans*. Isolated *Paracoccus* cpn60 shows very similar images to those of *Escherichia coli* tetradecameric cpn60, a seven-membered ring in the top view and a rectangular shape with four stripes in the side view. However, a small number of half-thick rectangles with two stripes are also seen which indicates that a single cpn60–heptamer ring has two stripes parallel to the plane of the ring. *Thermus* holo-chaperonin shows a bullet-like shape in the side view, and antibody against cpn10 binds only to the round side of the bullet. We conclude that a single cpn60–heptamer ring with two stripes stacks into two layers, and a cpn10 oligomer binds to one side of the layers.

Chaperonin; Holo-chaperonin; Electron microscopy; *Thermus thermophilus*

## 1. INTRODUCTION

Chaperonin is a novel class of proteins which facilitate folding of other proteins with the aid of ATP [1–3]. It includes two kinds of proteins with approximate molecular weights of 60 kDa (cpn60) and 10 kDa (cpn10). For *Escherichia coli*, they are also known as groEL and groES, respectively, and are purified separately, although they form a binary complex to be functional in the presence of ATP [4–11]. Native cpn60 has a very large molecular weight, 830–950 kDa, indicating that it exists as an oligomeric form [6–12]. Under the electron microscope, the native cpn60 shows a seven-membered ring in the top view and a rectangular shape with four clear stripes in the side view [6,7,12]. Based on these observations, it has been proposed that native cpn60 is a tetradecamer, $[cpn60]_{14}$, arranged in a cylinder form where a ring of seven cpn60 monomers, $[cpn60]_7$, stacks into two layers, and the four stripes are interpreted to be parallel to the axis of a cylinder, that is, perpendicular to the planes of two $[cpn60]_7$ rings [6,7,12]. However, based on the analyses of tilted images of a top view [13] and averaged side views [14], questions were raised

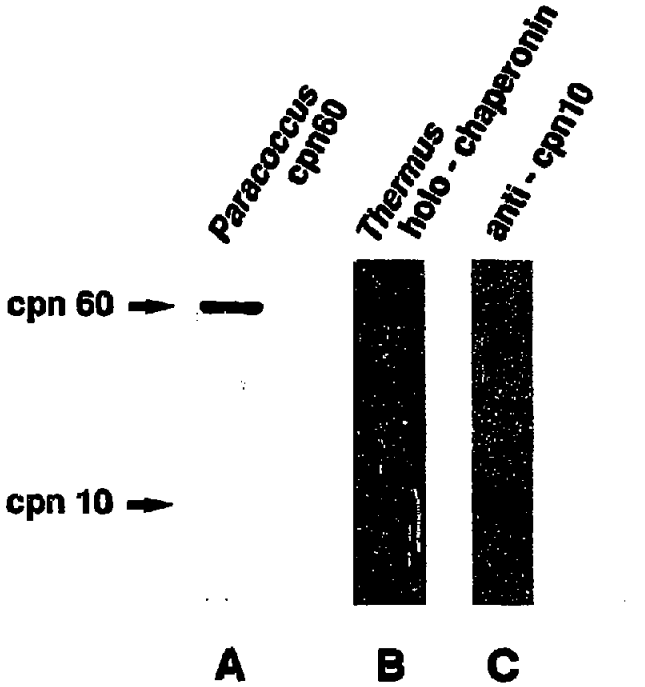


Fig. 1. Polyacrylamide gel electrophoreses of chaperonins in the presence of sodium dodecylsulfate. Concentration of the gels were 15%. (Lane A) The purified *Paracoccus denitrificans* cpn60. (Lanes B and C) The purified *Thermus thermophilus* holo-chaperonin. Approximately 15 μg of each protein were subjected to electrophoreses. Lane A is a gel stained with Coomassie brilliant blue R-250 and lane B was a membrane (Immobilon-P) stained with the same dye onto which proteins were blotted from the gel. Lane C was a blotted specimen which was subsequently treated with antibody against *Thermus thermophilus* cpn10 and visualized with horseradish peroxidase-conjugated anti- rabbit IgG antibody from donkey.

*Abbreviations:* cpn60 and cpn10, polypeptides of approximate molecular weights 60 and 10 kDa, respectively, which are included in holo-chaperonin; $[cpn60]_7$, a ring made from seven cpn60 monomers; $[cpn60]_{14}$, a tetradecamer of cpn60, stacked rings of two $[cpn60]_7$.

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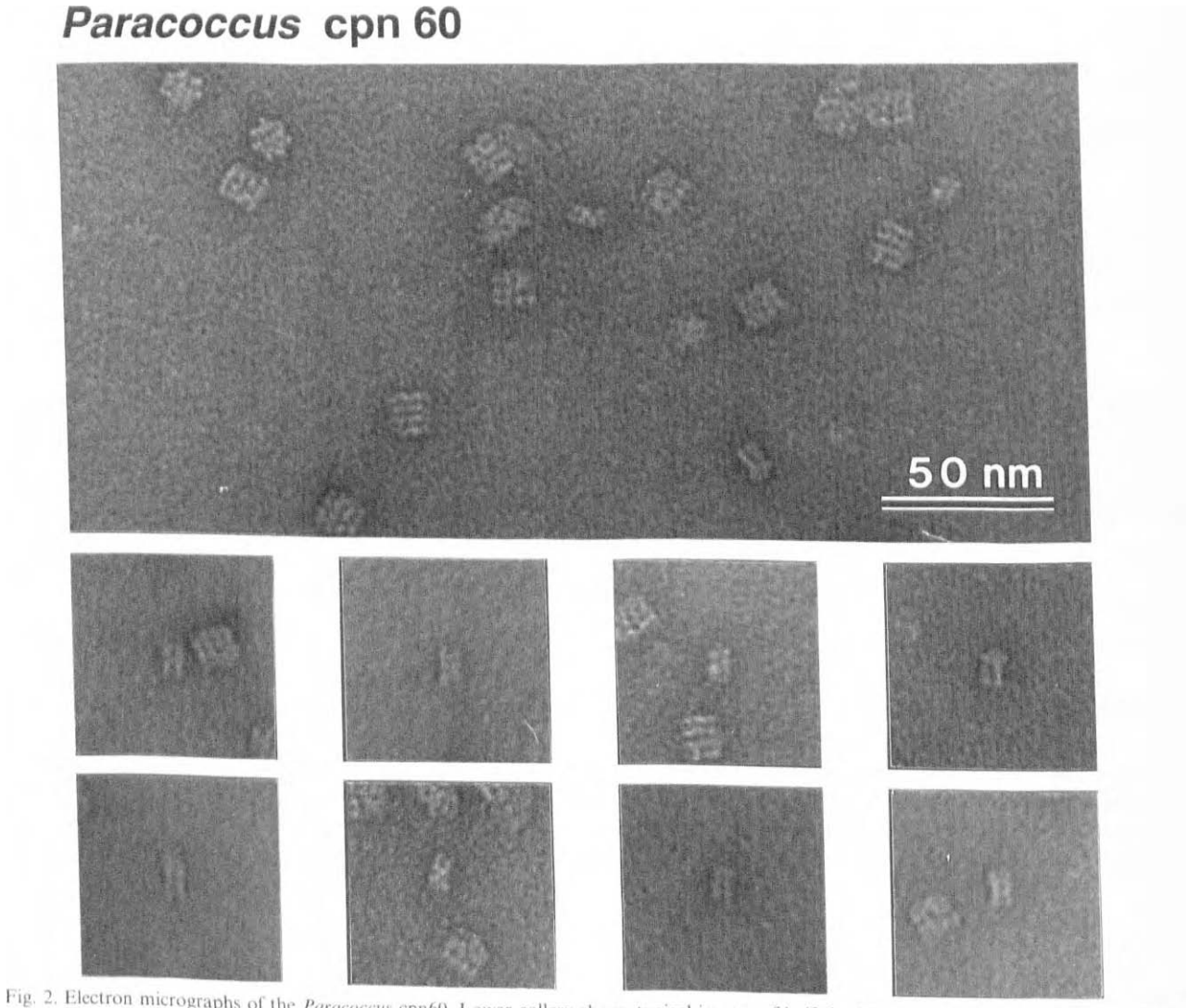


Fig. 2. Electron micrographs of the *Paracoccus* cpn60. Lower gallery shows typical images of half-sized rectangular particles with two stripes.

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recently against this interpretation of the stripes. It has been known that the native cpn10 is also a ring-shaped complex made from several cpn10 monomers [8] but the binding site of cpn10 to $[cpn60]_{14}$ is not known.

This paper is concerned with the interpretation of the four stripes observed in $[cpn60]_{14}$, and the binding site of cpn10 to $[cpn60]_{14}$. We have purified cpn60 from *Paracoccus denitrificans*, a non-photosynthetic purple bacterium the oxidative phosphorylation system of which is known to be very similar to that of mitochondria of eukaryotic cells. Electron microscopy revealed that most particles of *Paracoccus* cpn60 looked very similar to typical $[cpn60]_{14}$ but some particles showed a side view of a half-thick rectangle with two clear stripes. The structural model of $[cpn60]_{14}$ proposed in [6,7,12] is in obvious contradiction to this observation and should be modified accordingly. We have also purified a chaperonin from a thermophilic bacterium, *Thermus thermophilus*, and found that, in contrast to other sources such as bacteria [6,7,14–16], chloroplasts [17,18] and mitochondria [19–22], it is purified as a binary complex of cpn60 and cpn10 [23,24]. This has enabled us to observe the molecular shape of a 'holo-chaperonin' and, by using an antibody against cpn10, the location of cpn10 in holo-chaperonin was identified.

## 2. EXPERIMENTAL

### 2.1. *Purification of chaperonins*

Purification procedures of holo-chaperonin from *Thermus thermophilus* HB8 were described previously [23]. The same procedures were used for the isolation of cpn60 from *Paracoccus denitrificans* except for an insertion of Butyl Toyopearl column chromatography between DEAE cellulose and Sepharose CL-6B column chromatography. Fractions containing 58 kDa polypeptide eluted from a DEAE cellulose column were pooled, and solid ammonium sulfate was added to 20% saturation. This solution was applied to a Butyl Toyopearl

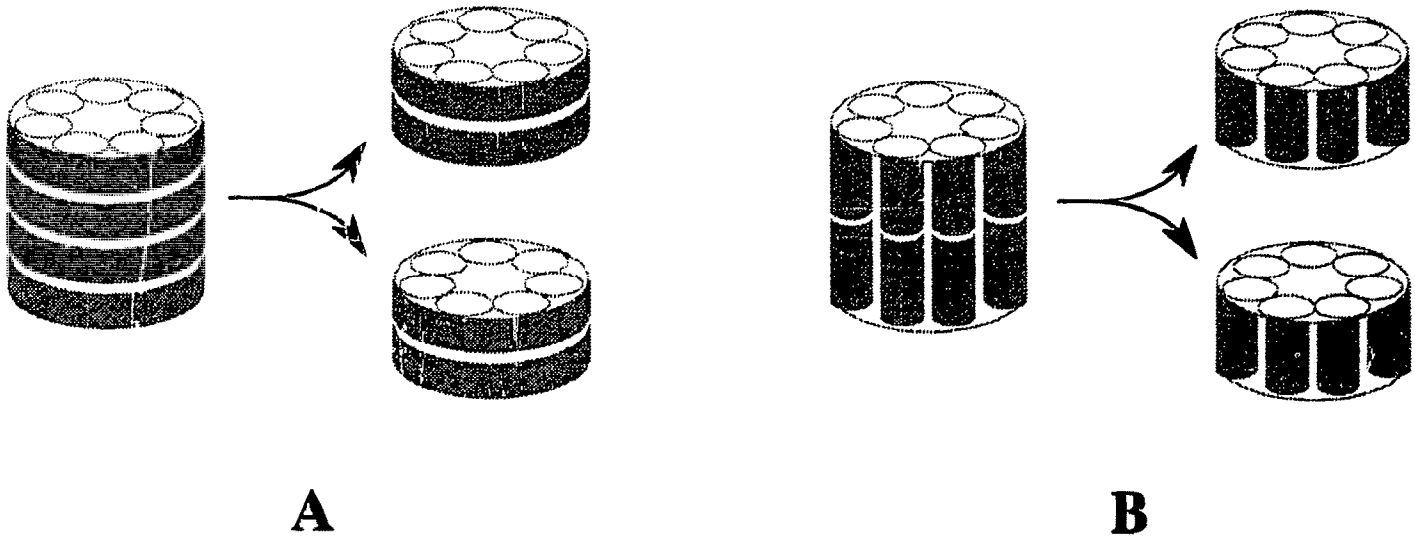


Fig. 3. Two possible directions of the stripes observed in electron micrographs of chaperonins. (A) A model proposed in this paper where stripes are parallel to the planes of the $[cpn60]_7$ rings. (B) A model proposed by Horn et al. and others where stripes are perpendicular to the planes of rings [6,7,12]. Both models agree in that two $[cpn60]_7$ rings stack into two layers and the whole shape of $[cpn60]_{14}$ is cylindrical. However, it should be noted that, when the stacked two rings dissociate into two single rings, a side view of each single ring should show two stripes (A) or four short stripes (B).

column equilibrated with 50 mM TRIS-$SO_4$, pH 8.0, 0.5 mM EDTA and 20% ammonium sulfate and was eluted with a linear gradient of 20–0% ammonium sulfate. Fractions containing 58 kDa polypeptide were pooled, concentrated by Amicon ultrafiltration, and applied to a Sepharose CL-6B column [23]. The 58 kDa polypeptide was eluted at void volume suggesting that it existed as a large oligomer. This purified fraction showed weak ATP hydrolyzing activity (0.03 $\mu$mol $P_i$ liberated per min at 37°C) and contained only 58 kDa polypeptide, not 10 kDa polypeptide (cpn10) as judged from polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (Fig. 1, lane A). The $NH_2$-terminal amino acid sequence of the 58 kDa polypeptide was determined to be AKEVKFDSDARDRMLKGVNILA-DAVKVTLGPXGXNVVIDXSFG (X represents an amino acid residue not determined unequivocally) which is very similar to *E. coli* cpn60, with 32 out of 40 amino acid residues identical, and thus the 58 kDa polypeptide was identified as *Paracoccus* cpn60. In fact, inhibition of spontaneous refolding of *Bacillus stearothermophilus* lactate dehydrogenase by the *Paracoccus* cpn60 was demonstrated (data not shown). This inhibition was not relieved by the addition of ATP, as expected, since it has been established that ATP-dependent facilitation of refolding requires cpn10 [10,11].

2.2. *Preparation of complexes of holo-chaperonin and antibody*

Both cpn60 and cpn10 polypeptide of *Thermus* holo-chaperonin was isolated with reverse-phase high performance column chromatography on a Bio-Rad $C_4$ column using 0.1% trifluoroacetic acid and increasing concentrations of acetonitrile as an elution solvent. Antisera against the purified cpn10 were raised in a rabbit by intradermal injections of 40 $\mu$g of protein. The antigen was dissolved in a solution of 50% (v/v) Freund's complete adjuvant. After 2 weeks an intravenous booster injection of 40 $\mu$g of protein was given. The IgG fraction of the antisera was purified with ammonium precipitation and chromatography on a DE-52 cellulose column (Whatman). The immunological specificity of the purified anti-cpn10 antibody was tested by immuno-blotting (Fig. 1, lane C). There was no detectable immunological cross-reaction with cpn60. *Thermus* holo-chaperonin and IgG at a molar ratio of about 1:20 were mixed in a volume of 0.1 ml and incubated for 24 h at 4°C. The mixture was passed through an acetylcellulose membrane and was subjected to gel filtration on a TSK G3000SWxl column with the elution buffer containing 20 mM TRIS-$SO_4$, 200 mM $Na_2SO_4$, pH 6.8. The fractions containing free holo-chaperonins and the complexes of holo-chaperonin and antibody preceded the peak of free antibodies. The former were pooled and examined by electron microscopy.

2.3. *Electron microscopy*

An aliquot of the sample solution was applied on an electron microscope specimen grid covered with a carbon support film which had been hydrophilized by ion bombardment. The excess of the solution was blotted with a filter paper. The specimen was immediately negatively stained with 1% Uranyl acetate for 2 min and placed on the stage of the electron microscope (JEOL JEM-1200EX). Images were recorded onto Fuji Electron Microscope Film at a magnification of 50,000 with an accelerating voltage of 100 kV.

## 3. RESULTS AND DISCUSSION

### 3.1. Paracoccus *cpn60*

Electron microscopic images of *Paracoccus* cpn60 are almost indistinguishable from those of *Escherichia coli* cpn60, namely a seven-membered ring in the top view and a rectangular shape with four stripes in the side view (Fig. 2). The diameter of the seven-membered ring, 13 nm, is very close to the values reported for *E. coli* $[cpn60]_{14}$, i.e. 12.9 nm [6] and 12.6 nm [7]. The dimension of a rectangle is 13.3 × 13.3 nm which is also close to the dimension of *E. coli* $[cpn60]_{14}$ (11.6 × 13.4 nm [6] and 10.2 × 12.4 nm [7]). Therefore, we conclude that *Paracoccus* cpn60 exists as the same oligomeric form as *E. coli* cpn60, a tetradecamer comprising two $[cpn60]_7$ rings. In addition to the typical images of $[cpn60]_{14}$, a small number of half-thick rectangles (13.3 × 6.5 nm) with two stripes were found (Fig. 2, gallery). They are

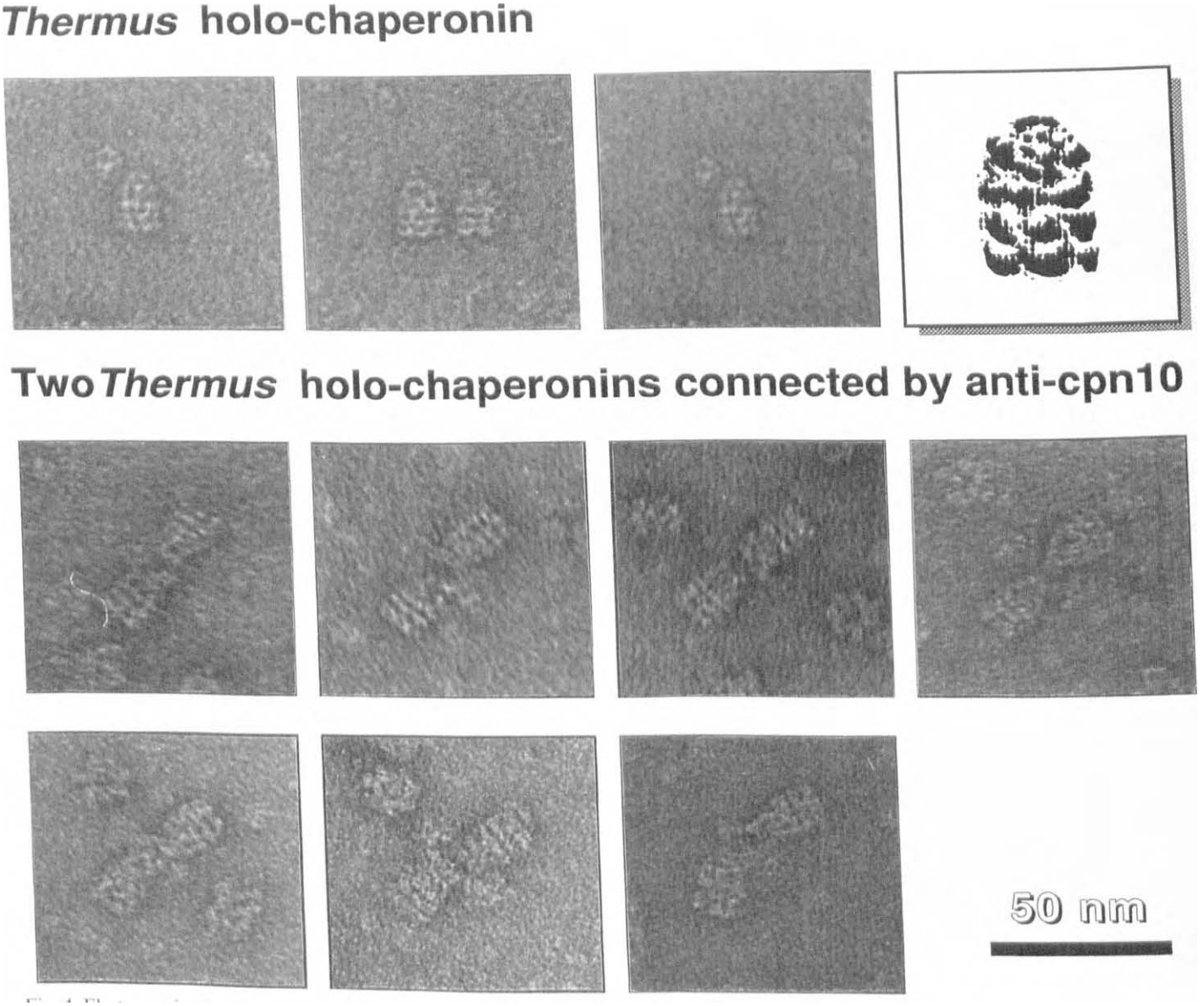


Fig. 4. Electron micrographs of *Thermus* holo-chaperonin untreated (upper gallery) and treated (lower gallery) with antibody against cpn10. The most right-hand figure of upper gallery is a schematic sketch of an image of *Thermus* holo-chaperonin. In the lower gallery, two holo-chaperonin molecules are connected by antibodies, and antibodies bind only around the top of the bullet-shaped holo-chaperonin.

certainly a side view of a single $[cpn60]_7$ ring. If the model of $[cpn60]_{14}$ structure proposed by Hohn et al. and other groups is correct, then four short stripes parallel to the shorter side of a rectangle should be observed for a single $[cpn60]_7$ ring (Fig. 3, model B) [6,7,12]. However, it is clear that two stripes run parallel to the longer side of rectangles. It is consistent to the model A in Fig. 3 where stripes are parallel to the plane of the $[cpn60]_7$ ring. Consequently, the four stripes observed in electron microscopic images of $[cpn60]_{14}$ run parallel to the planes of rings, rather than perpendicular. The reason why a single $[cpn60]_7$ ring shows two stripes is not known but a cpn60 monometer probably comprises two distinct domains each of which is seen as upper and lower stripes of $[cpn60]_7$.

### 3.2. Thermus *holo-chaperonin*

The *Thermus* holo-chaperonin contains both cpn60 and cpn10 (Fig. 1, lane B) [23]. Although a top view of this *Thermus* holo-chaperonin looks similar to that of *Paracoccus* cpn60 and cpn60s from other sources, a side view is not a simple rectangle but a bullet-like shape which has a round cap on one side of the rectangle (Fig. 4, upper gallery) [23,24]. It should also be noted that stripes are perpendicular to the axis of the bullet. Since a rectangular portion corresponds to $[cpn60]_{14}$, the cap

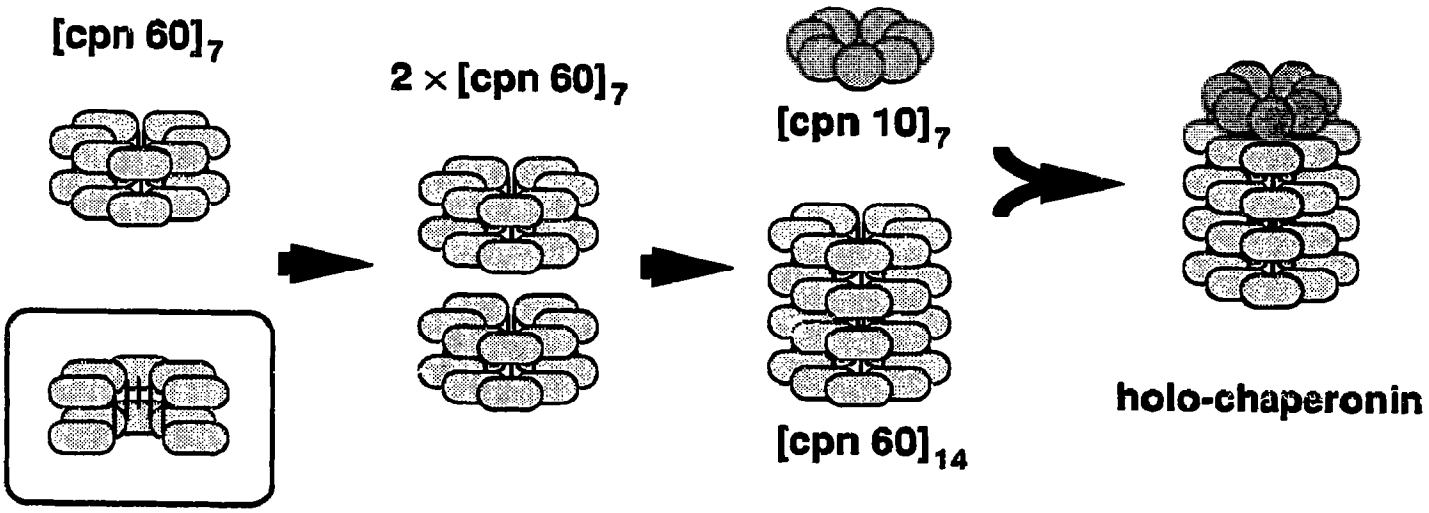


Fig. 5. Schematically illustrated structure of holo-chaperonin. A cpn60 monomer has two domains and a single cpn60 heptamer ring, denoted as $[cpn60]_7$, shows two stripes in the side view. Two $[cpn60]_7$ rings stack into two layers to form $[cpn60]_{14}$ and cpn10, which may also exist as a heptamer ring, binds only to the bottom or the top of cylindrical $[cpn60]_{14}$.

portion is probably occupied by cpn10. This was directly proved by immuno-electron micrograph using a antibody specific to *Thermus* cpn10. Two holo-chaperonin molecules are connected by antibodies only through the round tops of the bullet-shaped molecules (Fig. 4, lower gallery). In addition, there is no chain-like linear aggregate of holo-chaperonins connected by antibodies which should have been observed if cpn10s exist at both sides of holo-chaperonin.

### 3.3. *Structural model of holo-chaperonin*

These results lead us to propose a structural model of holo-chaperonin (Fig. 5). The main features of the model are as follows. First, a cpn60 monomer may consist of two domains which give rise to two stripes in a single $[cpn60]_7$ ring. This kind of two-domain structure has been reported for another molecular chaperone, hsc70, where an ATP-binding domain was isolated by limited proteolysis and was subjected to X-ray crystallography [25]. Secondly, two $[cpn60]_7$ rings stack into two layers and the thus formed $[cpn60]_{14}$ has four stripes parallel to the ring planes. This agrees with the suggestion by Hutchinson et al. [13] and Zwickl et al. [14] but disagrees with the model proposed by Hohn et al. and other groups [6,7,12]. Similar half-thick rectangular images with two stripes have been reported for cpn60 prepared from sperm mitochondria of *Heliothis virescens* [22]. Thirdly, cpn10, as an oligomeric ring form, as reported in [8], can bind only to one of two sides (bottom or top) of a cylindrical $[cpn60]_{14}$, making the side view of holo-chaperonin bullet-like. A similar bullet-like structure of the holo-chaperonin reconstituted from *E. coli* cpn60 and cpn10 was preliminarily reported [26]. Probably two $[cpn60]_7$ rings bind in a head-to-tail manner rather than in a tail-to-tail manner making the two sides of cylindrical $[cpn60]_{14}$ different each other. Although less likely, there remains another possibility, namely that binding of cpn10 to one side of $[cpn60]_{14}$ induces inhibition of cpn10 binding to another side. Since planes to two $[cpn60]_7$ rings and a cpn10 ring are all parallel in holo-chaperonin and the cpn10 ring may have seven cpn10 monomers, the seven-fold rotational symmetry of $[cpn60]_{14}$ may not be broken by binding of cpn10. This structure might be essential for the function of chaperonin but understanding the mechanism in molecular detail awaits further studies.

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